Biomolecular characterization of proteins involved in folate metabolism in *Plasmodium*

SYNOPSIS

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Introduction

Malaria is a leading infectious disease in sub-Saharan Africa (SSA), South-East Asia (SEA) and South America. Malarial infection is spread by *anopheles* mosquito infected with *Plasmodium* species, of which *Plasmodium falciparum* is most significant. There were 214million new cases of malaria and ~438 000 deaths worldwide in 2015 with the majority of them in children (3,06,000) under the age of five. This report is submitted by World Health Organisation in 2015.

Folates are vital co-factors in many biosynthetic pathways and blocking of folate synthesis leads to cell death. In humans, folate is obtained from dietary sources through a receptor-mediated salvage pathway. In *Plasmodium falciparum*, however, folate comes both from the salvage and *de novo* biosynthesis pathways**(Krungkrai*et al.*, 1989).**Folate pathway enzymesare inhibited by sulfadoxine (inhibits dihydropteroate synthase, DHPS) and pyrimethamine (inhibits dihydrofolate reductase, DHFR), used in Sulfadoxine-pyrimethamine (SP) combinations they were promising cocktails but were withdrawn from field due spread of resistant parasites. Five point mutations in DHPS (A437G, S436F/A, K540E, A581G and A613T/S) and two mutations in DHFR (N108 and R59) have been linked with resistance to SP therapeutic failure. Presently, SP is used as a partner drug of ACT artesunate-SP to treat uncomplicated *falciparum* malaria in many parts of the world which do not currently show SP resistance.

In *Plasmodium*, 6-hydroxymethyl-7,8,-dihydropterin pyrophosphokinase (HPPK) and dihydropteroate synthase (DHPS) are encoded as bi-functional HPPK-DHPS. *Plasmodium* bi-functional HPPK-DHPS is of ~700 residues and has fewer insertions when compared to the bacterial proteins (**Triglia and Cowman, 1994; Triglia*et al.*,1997).** The drug-resistant mutations were mapped on DHPS homology model and all these mutations, except for Ala581, occur in loop regions. However, the complete characterization and 3D crystal structure of *Plasmodium* bi-functional HPPK-DHPS is not yet available (**Hyde, 2005)**. In recent years, new leads have been developed against drug-resistant parasites based on combination of cocrystal structures, known inhibitors and lead candidates for both dihydrofolate reductase (DHFR)and the mitochondrial enzyme dihydroorotate dehydrogenase (DHOD) **(Baldwin*et al.*, 2005)**.

The artemisinin (ART) combination therapy (ACT) has been very successful, and (**WHO, 2006**) artemether-lumefantrine and artesunate- amodiaquine are the most widely used ACTs. Efforts to develop vaccine continue but have not been very successful. (**Coteron*et al.*, 2011; Yuthavong*et al.*, 2012)**. Resistance to all currently used antimalarial drugs has emerged and hence there is an urgent need to discover newer drugs (**Petersen*et al.*, 2011)**. Recently, polymorphisms in the propeller region of *P. falciparum*Kelch protein (k13 gene) have been found to be associated with ART resistance (**WHO, 2014) (Ariey*et al.*, 2014)**, and clinical resistance was observed in South East Asia and Sub-Saharan Africa which threatens ACT deployment. In India, artesunate-sulfadoxine/pyrimethamine (ASSP) is used for the treatment of uncomplicated *Plasmodium falciparum* infection (**Pornthanakasem*et al.*, 2016**). However, resistance to AS-SP has emerged in north-easternregion (NER) states. Therefore, artemether-lumefantrine (AL) is the recommended first line treatment for malaria in NER states **(Bharti*et al.*, 2016).**

In the present plan of work, we would like to perform structure and functional characterization of *plasmodium* enzyme/(s) involved in folate metabolism. For this, we will clone the gene without drug resistance mutations and will produce recombinant protein. Biochemical inhibition assays will be performed for different drugs after screening. Crystallization screening will be carried out in the presence or absence of substrate/inhibitors. Fragment-based drug discovery (FBDD), thermal shift assays (TSA) and co-crystallization experiments with commercially available analogs will also be performed.

Review of Literature

International status

Malaria remains a widespread human problem and several classes of drugs are used to treat it.However, resistance to all these drugs has been reported as reported by WHO published in the year 2015**.** *Plasmodium* bi-functional HPPK-DHPS is of ~700 residue and has several insertions when compared to bacterial counterparts. There have been few attempts towards characterization of *plasmodium* DHPS **(Triglia& Cowman, 1994; Triglia*et al.*, 1997).**

The *de novo* folate production pathway involves seven enzymes in plasmodium:
1. Guanine cyclohydrolase I (GCYH), **2.**Dihydroneopterin hydrolase (DHNTPase), **3.**Dihydroneopterinaldolase (DHNA) **4.** 6-hydroxymethyl-7,8,-dihydropterin pyrophosphokinase (HPPK) **5.** Dihydropteroate synthase (DHPS), **6.**Dihydrofolate synthase (DHFS) and **7.**Dihydrofolate reductase (DHFR, E.C. 1.5.1.3).

The four enzymes DHNA, HPPK and DHPS, DHFS are unique to micro-organisms and hence can be used as drug-targets. In some microorganism, these enzymes are encoded as bi- or trifunctional forms, though study of bi- (HPPK-DHPS and DHNA-HPPK) and trifunctional(DHNA-HPPK-DHPS) fusions has been more difficult. The 3D structures of mono-functionalenzymes DHNA, HPPK and DHPS are available for bacterial, fungal and from other sources.Structures of three bi-functional enzymes HPPK-DHPS of DHNA-HPPK-DHPS from *S.cerevisiae* **(Lawrence *et al.*, 2005)**; bi-functional HPPK-DHPS from *Francisellatularensis*; DHNA-HPPK from *Streptococcus pneumonia* **(Garçon*et al.*, 2006)**

The selectivity and specificity of inhibitors can be improved even against drug resistant parasites using structure-guided design, new chemical modifications and optimization of newer lead molecules **(Plouffe*et al.*, 2008; Verlinde*et al.*, 2009; Guiguemde*et al.*, 2010)**. Lead compounds that target multiple parasite species at multiple stages of the parasite life cycle are being actively sought (**Coteron*et al.*, 2011; Yuthavong*et al.*, 2012; Anthony *et al.*, 2012**). Recently, newer drugs have been developed for drug-resistant target dihydrofolate reductase (DHFR) (**Scott *et al.*, 2012)**anddihydroorotate dehydrogenase (DHOD) enzymes based on structure guided approaches (**Flannery *et al.*, 2013; Gamo, 2014;)** have also been reported.

The sulfadoxine drug inhibits DHPS, and clinical isolates reveal five point mutations against the drug. Thesemutations were mapped on DHPS homology model where all but Ala581 occur in loop regions (**Shaw *et al.*, 2014; Blaazer*et al.*, 2015).** However, the complete characterization and 3D crystal structure of *plasmodium* bifunctionalHPPK-DHPS is not yet known (**Pornthanakasem*et al.*,2016).** Further, fragment-based drug discovery (FBDD) approach has become mainstream in drug discovery projects **(Erlanson*et al.*, 2016; Benmansour*et al.*, 2017)**. This methodology has now been adopted widely in both thepharmaceutical industry and the academia.

National status

In India, several research groups (ICGEB, CSIR-CDRI, NII, MRC, RMRC and few University/IIT labs) are involved in the field of malaria parasitology and molecular biology. There are some medicinal chemistry groups (CDRI Lucknow, IICT Hyderabad, IISER-Pune) as well as who are involved in synthesis of parasite specific small molecule inhibitors. Presently, the structure determination of malarial parasite targets are being studied at IIT Bombay **(Bryksa*et al.*, 2011) and ICGEB (Banday*et al.*, 2014)** IISc, Bangalore **(Bharambe*et al.*, 2016)**, There is yet no concerted effort for biochemical characterization and structure determination of *plasmodium* enzymes involved in folate metabolism. Therefore, fragment-based screening after thermal-shift assays, combined with X-ray crystallographic screening for enzyme/(s) involved in folate metabolism shall be performed as per the present plan of work. For this, the gene without drug resistance mutations will be cloned and recombinant protein will be produced. The biochemical inhibitions assays will be carried out for different sulfa drugs. The crystallization screening will be carried out in the presence or absence ofsubstrate/inhibitors. An attempt will be made for fragment-based drug discovery (FBDD), thermal shift assays (TSA) and co-crystallization experiments.

Objectives

1. Cloning and production of wild type and drug-resistance mutant(s) of *Plasmodium* species bifunctional HPPK-DHPS enzyme.
2. Biophysical and biochemical characterization of enzyme substrate inhibitor complexes.
3. Crystallization and structure determination of wild type (WT) and drug-resistant (DR) mutant(s) bifunctional HPPK-DHPS with substrates/inhibitors.
4. Fragment-based drug discovery (FBDD) using thermal shift assays, cryo-soaking and co-crystallization experiments.

Strategy/ Methodology

Strategy for objective "1"

Cloning and production of wild type and drug-resistance mutant(s) of *Plasmodium* species bifunctional HPPK-DHPS enzyme:

Objective shall be achieved using techniques like PCR cloning, affinity chromatography, ion exchange chromatography and Gel filteration chromatography.

The full-length and resistant mutants of plasmodium HPPK-DHPS will be cloned into pETM11/pOPINF and the sequence will be verified by DNA sequencing. Rosetta(DE3)pLysSharbouring pETM11/pOPINF plasmodium HPPK-DHPS mutant variants will be cultured in LB supplemented along with their respective antibiotics until the cells grow to an optical density at 600 nm of ~1. Source of cloning vehicle is an express vector pOPINF (**https://www.addgene.org**) suitable for expression in *E. coli.* The expression of recombinant enzyme will induced by adding IPTG (isopropyl-**β**-dthiogalactopyranoside) to therecombinant bacterial culture and then the culture will bemaintained at 16°C overnight (16 to 20 h). Bacterial cells will be harvested by centrifugationat 3,000 × *g***for 10 min and stored at −20°C.** Ni-NTA affinity chromatography will be performed for the purification of recombinant protein. Protein purity and the subunit molecular mass will be analysed using SDS-PAGE. The native molecular mass of the recombinant plasmodium HPPK-DHPS will be determined by Superdex 200 HR 10/300 gel filtration chromatography. Mutants and wild type refer to the recombinant clones, which will be expressed in and purified from heterologous host *E.coli*for subsequent biophysical and structural studies. Sulphadoxine/pyrimethamine mutants of *Plasmodium*species reported previously, will be generated using site directed mutagenesis at specific amino acid positions of wild type recombinant clones.

Strategy for objective "2"

Biophysical and biochemical characterization of enzyme substrate- inhibitor complexes :

Objective "2" shall be achieved using techniques like real-time PCR

The biophysical characterization shall be performed by using techniques like Thermal shift assay/Isothermal titration calorimetry that helps in providing a full thermodynamic descriptionof binding events of enzyme-substrate interaction (**Groftehauge*et al.*, 2015)**. The HPPK-DHPSenzymes will be assayed using a modification of a published procedure (**Walter & Konigk,1980)**. The radiolabeled [3H/14C]para-aminobenzoic acid (pABA) will be purchased and theDHPS activity wild type and mutant enzymes will be performed by measurement of theconversion pABA to [^{3}H/^{14}C]dihydropteroate (DHP). Ki and Km values will also be calculated.

Strategy for objective "3"

Crystallization and structure determination of wild type (WT) and drug-resistant (DR)mutant(s) bifunctional HPPK-DHPS with substrates/inhibitors:

Objective "3" shall be achieved by using protein crystallization methodologies and X-raydiffraction

Highly purified (> 95% pure) and homogeneous fractions of HPPK-DHPS will be concentrated to higher concentrations and crystallization trial will be carried out via hanging drop or sitting drop methods to find ideal crystallization conditions of buffer systems. The purified WT and DR mutant enzymes will be crystallized in the presence of substrate(s) and drugs using the nano-drop dispensing robot, 96-well plate and hanging-drop vapour-diffusion method. In each drop, the protein solution will be mixed with crystallization solution. A single crystal of either the wild type or mutant enzyme will be harvested into a corresponding crystallizing solution containing appropriate

cryo-protectant briefly before being flash-frozen in liquid nitrogen.

Strategy for objective "4"

Fragment-based drug discovery (FBDD) using thermal shift assays, cryo-soaking andco-crystallization_experiments:

Objective "4" shall involve *in-silico* analyses

Crystals of the ligand-protein complexes will be produced by either soaking the ligand free crystals of HPPK-DHPS in the crystallization solution containing the substrates or co-crystallization in the presence of appropriate concentration of substrates. For fragment soakingexperiments, ~200-500 compound diversity fragment library will be purchased commercially and crystals will be transferred to 2 soaking drops of the reservoir mixture with 10% fragmentstock solution (100–500 mM in 100% DMSO). Crystals will be soaked between few min and 24 h at 20 °C, transferred to a cryo-protectant solution of the same ligand/reservoir solution containing 20% glycerol for a few seconds and flash-cooled in liquid nitrogen. Data will be collected at in-house X-ray or synchrotron radiation sources. The structures will be determined using the phasing or molecular replacement techniques with CCP4/PHENIX suites. A single crystal will be subjected to X-rays and a high-resolution data will be collected. The obtained data will be processed and scaled using HKL 2000 suit. Depending on the sequence identity of HPPK-DHPS, phases of native data will be obtained by molecular replacement method usingmolecular replacement based programs eg. MOLREP **(Vagin&Teplyakov, 1997),** PHASER-MR **(McCoy*et al.*, 2007)** BALBES **(Long*et al.*,2008)**or experimental phasing shall be deployed using anamalous scattering method and tools. Model building shall be carried out inCOOT **(Winn*et al.*, 2011).**

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